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Using Fluorogenic Peptide Substrates to Assay Matrix Metalloproteinases

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Abstract

A continuous assay method, such as one that utilizes an increase in fluorescence upon hydrolysis, allows for rapid and convenient kinetic evaluation of proteases. To better understand MMP behaviors and to aid in the design of MMP inhibitors, a variety of sequence specificity, phage display, and combinatorial chemistry studies have been performed. Results of these studies have been valuable for defining the differences in MMPs and for creating quenched fluorescent substrates that utilize fluorescence resonance energy transfer (FRET)/intramolecular fluorescence energy transfer (IFET). FRET triple-helical substrates have been constructed to examine the collagenolytic activity of MMP family members. The present chapter provides an overview of MMP and related FRET substrates and describes how to construct and utilize these substrates.

Keywords

FRET substrate; matrix metalloproteinase; triple-helical substrate; fluorogenic substrate; high-throughput screening

1. Introduction

1.1. Development of Peptide Substrates for Matrix Metalloproteinases

Catabolism of extracellular matrix (ECM) components has been ascribed to a family of Zn2+ metalloenzymes. These matrix metalloproteinases (MMPs) are important in connective tissue remodeling during development and wound healing. MMPs have also been implicated in a variety of disease states, including arthritis, glomerulonephritis, periodontal disease, tissue ulcerations, and tumor cell invasion and metastasis (1–4). MMPs are secreted as zymogens, and may be activated by plasmin, urokinase plasminogen activator, or other MMPs (5, 6). Many cells also express a number of MMP tissue inhibitors (tissue inhibitors of metalloproteinase; TIMP-1, TIMP-2, TIMP-3 and TIMP-4) (7). Thus, the presence of MMP protein is not necessarily indicative of MMP enzymatic activity. To investigate the *function* of MMPs, traditional methods such as ELISA and zymography are not ideal as they are often not capable of quantifying active proteases. In fact, zymography can activate inactive proteases, distorting quantification further. A continuous assay method, such as one

that utilizes an increase in fluorescence upon hydrolysis, allows for rapid and convenient kinetic evaluation of proteases, both in solution and cell surface bound. For this reason, significant research efforts have focused on designing substrates for MMP family members (8). Sequence specificity studies for MMPs have been performed with peptides based on protein sequences surrounding MMP cleavage sites, and the results of these studies have been comprehensively reviewed (9, 10). In addition, phage display and peptide libraries have been utilized to identify MMP-selective substrate sequences (11–17). Results of the aforementioned studies were subsequently used to design fluorogenic MMP substrates.

1.2. General Characteristics of Fluorogenic Substrates

Fluorogenic substrates provide a particularly convenient enzyme assay method, as they can be monitored continuously and utilized at reasonably low concentration ranges. There are three types of fluorogenic substrates: (i) aromatic amines, (ii) contact-quenched, and (iii) resonance energy transfer quenched (18). MMP fluorogenic substrates have been developed using resonance energy transfer quenching, which are sometimes referred to as intramolecular fluorescence energy transfer substrates (IFETS) (19).

1.2.1. Resonance Energy Transfer—Resonance energy transfer may occur between a fluorescent donor group and a quenching acceptor group when the fluorophore has a high quantum yield (Φ_F) and a fluorescence emission spectrum that is exactly overlapped by a strongly absorbing acceptor (quencher) (18). The efficiency of transfer will also depend upon the distance between the donor and acceptor (18, 19). Resonance energy transfer quenched fluorogenic substrates are thus created by incorporating the donor and acceptor on opposite sides of the scissile bond, at a distance which allows for highly efficient energy transfer. When the substrate is cleaved by enzyme, diffusion of the donor-containing substrate fragment away from the acceptor-containing substrate fragment results in loss of energy transfer and subsequent appearance of fluorescence. The creation of an optimal MMP fluorogenic substrate thus depends upon (i) incorporation of a fluorescent donor that has a high quantum yield, (ii) incorporation of an acceptor that absorbs at the donor fluorescence emission wavelength (λem), and (iii) the number of amino acid residues between the donor and acceptor.

1.2.2. Chemical Moieties Used for Donor/Acceptor Pairs—There are a wide variety of donor and acceptor groups that have been used in fluorogenic substrates (18, 19). MMP substrates have used one of eight different fluorophores. The first was Trp (20), which has ε_{280} = 5,600 $M^{-1}cm^{-1}$ and Φ_F = 0.2. The quencher group was *N*-2,4-dinitrophenyl (Dnp) (20, 21), which has an absorption maximum at λ = 363 nm and a prominent shoulder at λ = 410 nm (22). Alternatively, a fluorogenic substrate for the *Leishmania* surface metalloproteinase has used the dansyl group to quench Trp fluorescence (23). It should be noted that dansyl quenching of Trp decreases rapidly with intervening residue distance (18). Knight and colleagues proposed the use of (7-methoxycoumarin-4-yl)acetyl (Mca) as a fluorophore for MMP substrates (22). Mca has ε_{325} = 14,500 $M^{-1}cm^{-1}$ and Φ_F = 0.49. Mca is efficiently quenched by Dnp moieties (22, 24), as the shoulder in the Dnp absorption spectrum overlaps the Mca fluorescence emission spectrum (22). Dnp is also used as a quencher for MMP substrates containing the fluorophore *N*-methylanthranilic acid (Nma)

(25). 5-[(2-Aminoethyl)amino]naphthalene-1-sulfonic acid (Edans) has been used as a donor group for an MMP substrate (26). Edans has ε_{336} = 5,400 $M^{-1}cm^{-1}$ and Φ_F = 0.13. Edans fluorescence is quenched by the 4-(4-dimethylaminophenylazo)benzoic acid (Dabcyl) group (26). MMP substrates have been developed with Lucifer Yellow (LY) as a fluorophore (27, 28). LY has ε_{420} = 12,000 $M^{-1}cm^{-1}$ and is quenched by 5-carboxytetramethylrhodamine (Ctmr) (27). MMP and ADAM (a disintegrin and metalloproteinase domain) substrates have utilized 5-carboxyfluorescein (Fam) as a fluorophore (29, 30). Fam has ε_{492} = 65,000 $M^{-1}cm^{-1}$ and Φ_F = 0.92. Dabcyl was used for quenching Fam in ADAM substrates (30), while QXL 520 was the Fam quencher in MMP substrates (29). 2-Aminobenzoyl (Abz)/anthranilamide has been applied as a fluorophore for solution and solid-phase MMP-9 and MMP-12 substrates used for screening phosphinic peptides as inhibitor (31, 32). Abz has ε_{316} = 2,300 $M^{-1}cm^{-1}$ and Φ_F = 0.60 (33–35). Tyr(NO_2) was used to quench Abz fluorescence (31, 32). Lastly, Cy3 was used as fluorophore and Cy5Q as quencher for an MMP-3 high-throughput screening (HTS) substrate (36). Cy3 has ε_{550} = 150,000 $M^{-1}cm^{-1}$ and Φ_F = 0.15.

1.2.3. Practical Aspects of Donor/Acceptor Groups—Trp and Tyr(NO_2) can be incorporated easily by solid-phase peptide methodology and either Dnp or dansyl is acylated to the *N*-terminus of the peptide. Other moieties require additional synthetic steps. For example, to use the Mca/Dnp pair, a Dnp derivative such as *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionic acid (Dpa) (22) or Lys(Dnp) (24) must be synthesized (see below) and incorporated by solid-phase methods, while Mca is acylated to the peptide *N*-terminus. Alternatively, the Mca derivative L-2-amino-3-(7-methoxy-4-coumaryl)propionic acid (Amp) can be synthesized (see below) and incorporated by solid-phase methods (37). For Dabcyl/Edans substrates, derivatives of Glu(Edans) must be prepared (see below). Substrates containing Nma, Fam, or Cy3 can be synthesized using “on-resin” reactions to create Lys(Nma), Lys(Fam), or Lys(Cy3) moieties (see below).

In contrast to solid-phase assembly of fluorogenic substrates, “post-synthesis” approaches have been used for the preparation of peptides containing the LY/Ctmr pair (27, 28) or Cy5Q (36). Although they are more laborious, post-synthesis approaches do alleviate one potential purification problem of fluorogenic peptides. Typically, fluorophores and quenchers are hydrophobic, and thus the reverse-phase (RP) HPLC separation of the desired peptide from deletion peptides containing these groups can be difficult.

1.3. Fluorogenic Substrates for Matrix Metalloproteinases

1.3.1 Single-Stranded Substrates—Stack and Gray developed the first fluorogenic MMP substrate, Dnp-Pro-Leu-Gly~Leu-Trp-Ala-D-Arg-NH2 (20). This substrate was hydrolyzed at the Gly-Leu bond by MMP-1 and MMP-2 (20) (Table 1). The activities of the full length and the *C*-terminally-truncated MMP-1 and MMP-3 have also been compared toward the substrate Dnp-Pro-Leu-Gly~Leu-Trp-Ala-D-Arg-NH2 (38). The kcat/KM values for MMP-1 hydrolysis of this substrate do not change significantly even if the enzyme lacks the *C*-terminal domain (Table 1). *C*-terminal truncation also has little effect on MMP-3 hydrolysis of this peptide (Table 1). MMP-1 and MMP-3 peptidase specificity appears to be

determined by the catalytic domain, with only a small, if any, contribution from the *C*-terminal hemopexin-like domain (except for triple-helical substrates; see below).

The fluorogenic substrate Dnp-Pro-Leu-Gly~Leu-Trp-Ala-D-Arg-NH_2, when dissolved in trifluoroethanol, shows a significant change in structure (as monitored by circular dichroism spectroscopy) in the presence of Ca^{2+} (39). These authors have suggested a role for Ca^{2+} in the binding and hydrolysis of substrate by MMP-1 (39). In general, it has been noted that several peptide substrates of MMP-1 or MMP-2 bind Ca^{2+}, resulting in structural changes in the substrate (39, 40).

Combining the principle of Stack and Gray's substrate with MMP-1, MMP-3, and MMP-8 specificity studies, Fields proposed Dnp-Pro-Tyr-Ala~Leu-Trp-Ala-Arg-NH2 as an MMP-1 substrate, Dnp-Pro-Tyr-Ala~Tyr-Trp-Met-Arg-NH2 as an MMP-3 substrate, and Dnp-Pro-Leu-Ala~Tyr-Trp-Ala-Arg-NH2 as an MMP-8 substrate (41). In similar fashion, MMP-2 and MMP-9 specificity studies (42) were used to design the substrate Dnp-Pro-Leu-Gly~Met-Trp-Ser-Arg (43). With the exception of MMP-8 substrates Dnp-Pro-Leu-Ala~Leu-Trp-Ala-Arg and Dnp-Pro-Leu-Ala~Tyr-Trp-Ala-Arg, these "optimized" substrates were not very active (43) (Table 1). Subsequent elongation of the MMP-2 and MMP-9 substrates to include P_5 and P_5' subsite residues (44) may enhance their activity and selectivity. Niedzwiecki et al. (21) developed Dnp-Arg-Pro-Lys-Pro-Leu-Ala~Nva-Trp-NH_2 and Dnp-Arg-Pro-Lys-Pro-Leu-Ala~Phe-Trp-NH_2 as MMP-3 substrates (Table 1).

Knight and colleagues designed Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH2, which, at the time, proved to be the best substrate for MMP-1, MMP-2, MMP-7 and MMP-9 (22) (Table 1). Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH2 was also a good substrate for MMP-3 (22) (Table 1). Subsequent studies have shown this substrate to be hydrolyzed by MMP-8, MMP-12, MMP-13, MMP-14, MMP-26, and *C*-terminally-truncated MMP-13, MMP-14, MMP-17 and MMP-25 (45–50) (Table 1). As was the case in the prior study of MMP-1 and MMP-3 (38), the kcat/KM values for MMP-13 hydrolysis of Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH2 did not change significantly even if the enzyme lacked the *C*-terminal domain (46). However, some differences were noted for Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH2 hydrolysis by MMP-14 with and without the *C*-terminal domain (50) (Table 1).

The Knight substrate was extended to include Lys in the P_4 subsite, resulting in Mca-Lys-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH2 (51). The modified substrate had improved solubility and greater activity towards a variety of MMPs (51) (Table 1). The Mca/Dpa pair was subsequently used for Mca-Pro-Cha-Gly~Nva-His-Ala-Dpa-NH_2 (where Cha is 3-cyclohexylalanine), which proved to be an efficient substrate for MMP-1, MMP-8, and MMP-13 (45, 46) (Table 1).

Nagase et al. (24) designed several fluorogenic substrates based on the Mca/Dnp combination (NFF-1, NFF-2 and NFF-3), one of which was selectively hydrolyzed by MMP-3. NFF-3 [Mca-Arg-Pro-Lys-Pro-Val-Glu~Nva-Trp-Arg-Lys(Dnp)-NH2] was hydrolyzed rapidly by MMP-3 (kcat/KM = 218,000 sec-1M-1) and very slowly by MMP-9 (kcat/KM = 10,100 sec-1M-1), but there was no significant hydrolysis by MMP-1, MMP-2 or MMP-7 (9, 24) (Table 1). NFF-3 was the first documented synthetic substrate hydrolyzed

by only certain members of the MMP family, and thus has important application for the discrimination of MMP-3 activity from that of other MMPs. For high-throughput screening (HTS) of potential MMP-3 inhibitors, NFF-3 was modified by replacement of Mca/Dnp with Cy3/Cy5Q, resulting in acetyl-Arg-Pro-Lys(Cy3)-Pro-Val-Glu~Nva-Trp-Arg-Lys(Cy5Q)-NH_2 (36).

The fluorogenic substrate Dnp-Pro-Cha-Gly~Cys(CH3)-His-Ala-Lys(Nma)-NH2 was found to be hydrolyzed rapidly by the 19 kDa MMP-1 and MMP-9 (25) (Table 1). Dabcyl-Gaba-Pro-Gln-Gly~Leu-Glu(Edans)-Ala-Lys-NH_2, where Gaba is γ-amino-n-butyric acid, is a very good substrate for MMP-1, MMP-2, MMP-3 and MMP-9 (26) (Table 1). The combination of LY as fluorophore and Ctmr as quencher has been used for the assembly of the MMP-1 substrate LY-Gly-Pro-Leu-Gly~Leu-Arg-Ala-Lys(Ctmr) (27) (Table 1). The Abz/Tyr(NO_2) pair has been applied for the substrates Abz-Gly-Pro-Leu-Gly~Leu-Tyr(NO_2)-Ala-Arg-NH_2 for MMP-9 (31) and Ala-Tyr(NO_2)-Gly-Pro-Leu-Gly~Leu-Tyr-Ala-Arg-Lys(Abz)-Gly for MMP-12 (32). Finally, the Fam/Dabcyl pair has been used to develop several substrates that discriminate between MMPs and related metalloproteinases (30) (see below).

1.3.2 Triple-Helical Substrates—Triple-helical peptide (THP) models of the collagenase cleavage site in type I collagen were constructed in order to further examine the substrate specificity of MMP family members. The α1(I)772–786 THP was hydrolyzed by MMP-1, MMP-1(Δ243–450), MMP-2 and MMP-13 but not by MMP-3 or MMP-3(Δ248–460) (52, 53). Sequence and mass spectrometric analysis of the products generated by the MMPs indicated that MMP hydrolysis occurred primarily at the Gly-Ile bond, which is analogous to the bond cleaved in native type I collagen. Thus, the α1(I)772–786 THP contained all necessary information to direct MMP-1, MMP-2 and MMP-13 binding and proteolysis. Similar results were reported by Ottl et al. (54–56), who found that a heterotrimeric THP incorporating the [α1(I)]2α2(I)772–783 sequence was efficiently hydrolyzed by MMP-8.

To measure collagenolytic MMP activities, fluorogenic triple-helical peptides (fTHPs) have been developed. These triple-helical substrates utilize FRET primarily via incorporation of Mca as the fluorophore and the Dnp as the quencher within the same peptide chain (57–59). Typically, the P_5' position of the fTHP accommodates Lys(Dnp) while the P_5 position accommodates Lys(Mca) (seenote 1). fTHPs have subsequently provided insights into (a) the relative triple-helical peptidase activities of the various collagenolytic MMPs, (b) the collagen preferences of these MMPs, and (c) the relative roles of MMP domains and specific residues in efficient collagenolysis (50, 52, 53, 57, 58, 60–66). fTHPs have been noted as being particularly advantageous for dissecting the mechanism of collagenolysis (8).

Due to the differing sequences of the fTHPs, their relative triple-helical thermal stabilities can vary (64, 65). In order to obtain fTHPs that are stable under near-physiological

[1]In another example of an fTHP, Trp was the fluorophore and the dansyl (Dns) group was used as the quencher (110). A single Trp was incorporated at the *N*-terminus of one chain (α1′), while a single Dns group was incorporated at the *N*-terminus of a second chain (α1). Unfortunately, the quenching of Trp fluorescence was inefficient. Although the substrate was cleaved, kinetic parameters were not reported, presumably due to the high fluorescent background of the substrate.

conditions, as well as to examine the effects of substrate thermal stability on MMP activity, the “peptide-amphiphile” approach has been utilized (67–70). Peptide-amphiphiles (PAs) mimic defined topological structures by incorporating an amino acid sequence with the propensity to form a triple-helix as the polar head group and a dialkyl or monoalkyl hydrocarbon chain as the non-polar tail (67–70). Desirable peptide head group melting temperature (T_m) values can be achieved for *in vivo* use, as triple-helical PAs have been constructed with T_m values ranging from 30 to 70°C (52, 53, 57, 68, 69, 71–73). fTHP PAs have been created that, based on their T_m values, are suitable for studying MMP activity under *in vivo* conditions.

A series of fTHPs has been assembled that incorporate sequences based on a consensus types I-III collagen 769–783 region (Table 1). The fTHP substrates have been utilized to determine individual kinetic parameters (Table 1) and activation energies for hydrolysis of triple-helices (57, 58, 61, 63–65). MMP-1, MMP-2, MMP-8, MMP-9, MMP-13 and MMP-14 hydrolysis of the consensus types I-III collagen sequence (designated fTHP-3 and fTHP-4) occurred at the Gly~Leu bond, the analogous bond cleaved by these MMPs in the native collagen chains (6). Thus, the incorporation of the fluorophore/quenching pair of Mca and Dnp did not affect the ability of the enzyme to recognize or cleave the substrate. A modified version of the consensus types I-III collagen fTHP, in which the P_1' subsite incorporated a Cys(4-methoxybenzyl) group (C_{10}-fTHP-9), was hydrolyzed efficiently by MMP-14, but poorly by MMP-1, MMP-2, MMP-3 or MMP-9 (63).

MMP selectivity has been observed with a type V collagen derived fTHP. The α1(V)436–447 fTHP was hydrolyzed efficiently by MMP-2, MMP-9 and MMP-12 (Table 1), but was not hydrolyzed by MMP-1, MMP-3, MMP-13 or MMP-14 (61, 74).

1.3.3 Fluorogenic Substrates for High-Throughput Screening (HTS)—The Mca fluorophore is compatible with commercial plate readers, and thus HTS for MMPs has been previously established using FRET substrates with Mca as fluorophore and Dnp as quencher (75–77). For example, Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2 was used for screening a 324 member library of dipeptides acylated with isobutyl succinate (75), a combinatorial library of Cys-diketopiperazines (76), and a combinatorial library of phosphinic peptides (77, 78).

Inhibitor fingerprinting of MMPs was performed by screening 1400 peptide hydroxamates with three different substrates: Dabcyl-Gaba-Pro-Gln-Gly~Leu-Glu(Edans)-Ala-Lys-NH_2 for MMP-3, MMP-9 and MMP-14; Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2 for MMP-2 and MMP-7; and Mca-Pro-Cha-Gly~Nva-His-Ala-Dpa-NH_2 for MMP-8 and MMP-13 (79). Ala-Tyr(NO_2)-Gly-Pro-Leu-Gly~Leu-Tyr-Ala-Arg-Lys(Abz)-Gly was used for solid-phase screening of MMP-12 against a 165,000 member phosphinic peptide library (32, 80).

One problem with FRET-based assays is that compounds being screened may have absorption maxima that coincide with the emission wavelength of the fluorophore. This results in quenching of fluorescence by the compound and an incorrect designation as an inhibitor. Alternatively, fluorescent compounds that have similar excitation and emission maxima as the fluorophore will fluoresce during the assay, and may not be recognized as

inhibitors. As noted by George et al. for HTS of MMP-3, the CyDye pair of Cy3/Cy5Q [for the substrate acetyl-Arg-Pro-Lys(Cy3)-Pro-Val-Glu~Nva-Trp-Arg-Lys(Cy5Q)-NH_2] was much less susceptible to false results than the Mca/Dnp pair, as <1% of a random library were auto-fluorescent at Cy3 wavelengths while >10% of the same library could not be screened using Mca/Dnp due to auto-fluorescence and interference (36).

fTHP assays have been utilized with 96-, 384-, and 1536-well plates (57, 58, 60–65, 81–83). Because the THPs have distinct conformational features that interact with protease secondary binding sites (exosites) (82), these substrates can be utilized for the identification of non-active site binding inhibitors. Exosites have been shown to represent unique opportunities for the design of selective inhibitors (84, 85). Initial clinical trials with MMP inhibitors were disappointing, with one of the problematic features being a lack of selectivity (1, 86, 87). Selective exosite-binding inhibitors for MMPs could represent a potential next generation in metalloproteinase therapeutics.

The THP substrate fTHP-15 [$(Gly\text{-}Pro\text{-}Hyp)_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly~Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-$(Gly\text{-}Pro\text{-}Hyp)_5$-$NH_2$] has been utilized for screening of a 65,000 member compound library against MMP-13 in a 1536-well format (83). Several novel lead compounds were identified.

1.4 Fluorogenic Substrates for Metalloproteinases Related to MMPs

The **a** **d**isintegrin **a**nd **m**etalloproteinase (ADAM) family has been recognized for their activities in shedding cell surface proteins. A variety of fluorogenic substrates have been described for ADAM17 (tumor necrosis factor-alpha converting enzyme; TACE), including Mca-Lys-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH2, Mca-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Ala-Arg-NH2, Dabcyl-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Ala-Arg-Edans, Dabcyl-Pro-Cha-Gly~Cys(CH3)-His-Ala-Lys(Fam)-NH2 and Dabcyl-Leu-Ala-Gln-Ala-Homophe-Arg-Ser-Lys(Fam)-NH_2 (30, 51) (Table 1). Dabcyl-His-Gly-Asp-Gln-Met-Ala-Gln-Lys-Ser-Lys(Fam)-NH_2, in addition to Dabcyl-Pro-Cha-Gly~Cys(CH3)-His-Ala-Lys(Fam)-NH2 and Dabcyl-Leu-Ala-Gln-Ala-Homophe-Arg-Ser-Lys(Fam)-NH_2, are substrates for ADAM8, ADAM10 and ADAM12 (30). Dabcyl-His-Gly-Asp-Gln-Met-Ala-Gln-Lys-Ser-Lys(Fam)-NH_2 is greatly favored by ADAM8 compared with the other tested ADAM family members (30), whilst Dabcyl-Pro-Cha-Gly~Cys(CH3)-His-Ala-Lys(Fam)-NH2 is preferred by MMPs over ADAM family members (30) (Table 1).

FRET substrates have also been developed for members of the **a** **d**isintegrin **a**nd **m**etalloproteinase with **t**hrombo**s**pondin motifs (ADAMTS) family (66, 88–90). An ADAMTS-4/ADAMTS-5 substrate was designed that incorporated the aggrecan 1480–1481 cleavage site within a triple-helical structure. Residues 1475–1484 from bovine aggrecan were sandwiched between 5 Gly-Pro-Hyp repeats at the *N*- and *C*-termini. Lys(Mca) and Lys(Dnp) were substituted for Ala and Ile in the P_5 and P_5' subsites, respectively, resulting in fTHPa [C_6-$(Gly\text{-}Pro\text{-}Hyp)_5$-Gly-Thr-Lys(Mca)-Gly-Glu~Leu~Glu~Gly-Arg~Gly-Thr-Lys(Dnp)-Gly-Ile-Ser-$(Gly\text{-}Pro\text{-}Hyp)_5$-$NH_2$] (66). Circular dichroism spectroscopy indicated a triple-helical structure. A substrate analogous to fTHPa was designed of the same length and composition but without the ability to form a triple-helix [fSSPa; C_6-$(Gly\text{-}Pro\text{-}Hyp\text{-}Pro\text{-}Hyp\text{-}Gly)_2$-Gly-Pro-Hyp-Gly-Thr-Lys(Mca)-Gly-Glu~Leu~Glu~Gly-

Arg~Gly-Thr-Lys(Dnp)-Gly-Ile-Ser-(Gly-Pro-Hyp-Pro-Hyp-Gly)$_2$-Gly-Pro-Hyp-NH_2] (66). In this case, the Gly, Pro and Hyp residues at the *N*- and *C*-termini were scrambled. The circular dichroism spectrum for fSSPa was indicative of a polyPro II helix, but not a triple-helix. Both fTHPa and fSSPa were readily hydrolyzed by ADAMTS-4 and ADAMTS-5, and individual kinetic parameters determined (Table 1). fSSPa was utilized for screening of a compound library (n = 960) against ADAMTS-4 in a 384-well format (91).

Additional ADAMTS-4 and ADAMTS-5 FRET substrates have been obtained from phage display screening (89, 90, 92) (Table 1). Lys(6-Fam)-Asp-Val-Gln-Glu-Phe-Arg-Gly-Val-Thr-Ala-Val-Ile-Arg-Cys(Qsy-9)-Lys-Gly-Lys was initially described as an ADAMTS-4 selective substrate (89), but was later shown to be hydrolyzed by ADAMTS-5 (93). The use of 6-Fam and Qsy-9 lead to a significant inner filter effect and subsequent difficulties in kinetic evaluation of the substrate, limiting its use as a HTS tool (89). Fam-Ala-Glu~Leu-Gln-Gly-Arg-Pro-Ile-Ser-Ile-Ala-Lys(Tamra) was a sensitive substrate for ADAMTS-4 and its processed variants (90) (Table 1). Unlike fTHPa and fSSPa, these ADAMTS-4/ADAMTS-5 substrates are most likely too small to use for identifying exosite inhibitors (92). A FRET substrate for ADAMTS-13 utilized residues Asp1596-Arg1668 of von Willebrand factor with Nma in the P_7 subsite (residue 1599) and Dnp in the P_5' subsite (residue 1610) (88). Due to its size the ADAMTS-13 FRET substrate may be useful for screening exosite inhibitors (88).

2. Materials

2.1. Synthesis of Derivatives Used in Fluorogenic Substrates

1. Fluoren-9-ylmethoxycarbonyl *N*-hydroxysuccinimide ester (Fmoc-NHS ester).
2. Dimethoxyethane.
3. Lys(Dnp).
4. 10% aqueous Na2CO3.
5. Concentrated HCl.
6. Ethyl acetate.
7. $CHCl_3$.
8. Silica gel 60 (Merck 9385).
9. $CHCl_3$–methanol–acetic acid: 9.25:0.5:0.25 v/v.
10. Fmoc-Gly.
11. 1-hydroxybenzotriazole (HOBt).
12. 4-dimethylaminopyridine (DMAP).
13. Dichloromethane (DCM).
14. *N*,*N*-dimethylformamide (DMF).
15. **Fmoc-Lys(Dnp)-Gly-4-hydroxymethylphenoxy (**HMP) resin.

16. *N*,*N*′-diisopropylcarbodiimide (DIPCDI).
17. Piperidine.
18. Fmoc-4-(2′,4′-dimethoxyphenylaminomethyl)phenoxy (DMPAMP) resin.
19. Fmoc-Asn.
20. Pyridine.
21. [Bis(trifluoroacetoxy)iodo]benzene.
22. Diethyl ether.
23. Solid Na_2CO_3.
24. Ethanol.
25. 1-fluoro-2,4-dinitrobenzene.
26. (2*R*)-borane-10,2-sultam glycinate (Oxford Asymmetry, Abingdon).
27. 4-bromomethyl-7-methoxycoumarin (Fluka).
28. NBu_4HSO_4.
29. $LiOH{\bullet}H_2O$.
30. $MgSO_4$.
31. Tetrahydrofuran.
32. Edans (Sigma).
33. Boc-Glu-OBzl.
34. (2*R*,3*S*)-(–)-6-oxo-2,3-diphenyl-4-morpholine-carboxylate (Aldrich).
35. Sodium bis(trimethylsilyl)amide.
36. $PtCl_2$.

2.2. Synthesis of Fluorogenic Substrates

1. Benzotriazolyl *N*-oxytrisdimethylaminophosphonium hexafluorophosphate (BOP).
2. *N*,*N*-Diisopropylethylamine (DIEA).
3. Glacial acetic acid.
4. $KHSO_4$.
5. Benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP).
6. *N*-methylmorpholine (NMM).
7. 7-methoxycoumarin-4-acetic acid.
8. 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HBTU).

9. *p*-(*p*-dimethylaminophenylazo)benzoic acid (Sigma).
10. *N*-methylpyrrolidone (NMP).
11. Thiophenol.
12. Succinimidyl *N*-methylanthranilate (Molecular Probes, Eugene, OR).
13. Hydrofluoric acid (HF).
14. Trifluoroacetic acid (TFA).
15. 0.025 M sodium phosphate, pH 7.0.
16. NaOH, 1M.
17. $NaIO_4$: 0.04M dissolved in 0.025 M sodium phosphate, pH 7.0 (final pH of the periodate solution, pH 6.7, not further adjusted).
18. Lucifer Yellow CH (Molecular Probes, Eugene, OR): 0.03M in 0.1 M sodium acetate, pH 4.5
19. 5-carboxytetramethylrhodamine succinimidyl ester (Molecular Probes, Eugene, OR).
20. Acetonitrile.
21. Triisopropylsilane (TIS).
22. Cy3 mono acid.
23. 7-Azobenzotriazolyoxytris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP).
24. Acetic anhydride.
25. Cy5Q *N*-hydroxysuccinimide ester (Cy5Q NHS ester).
26. Fmoc-Lys(Mca) (EMD Biosciences).

2.3. Assaying Matrix Metalloproteinases Using Fluorogenic Substrates

1. Dimethylsulfoxide (DMSO).
2. Assay buffer A: 0.5 M Tris-HCl pH 7.7, 5 mM $CaCl_2$, 0.2 M NaCl, up to 20% DMSO.
3. Assay buffer B: 0.1 M HEPES pH 7.5, 10 mM $CaCl_2$.
4. Assay buffer C: 50 mM Tris-HCl pH 7.5, 0.15 M NaCl, 10 mM CaCl2, 0.05% Brij 35, 0.02% NaN3.
5. Assay buffer D: 50 mM sodium acetate buffer, pH 6.0.
6. Assay buffer E: 0.1 M Tris-HCl pH 7.5, 0.1 M NaCl, 10 mM $CaCl_2$, 0.05% Brij 35.
7. Assay buffer F: 50 mM Tris-HCl pH 7.6, 0.15 M NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.01% Brij 35.

8. Assay buffer G: 50 mM Tris-HCl pH 7.6, 0.2 M NaCl, 5 mM $CaCl_2$, 20 μM $ZnSO_4$, 0.05% Brij 35.
9. Assay buffer H: 50 mM Tris-HCl pH 7.7, 0.15 M NaCl, 5 mM $CaCl_2$.
10. Enzyme assay buffer (EAB); 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij 35.

3. Methods

3.1. Methods For the Synthesis of Derivatives Used in Fluorogenic Substrates

3.1.1. Synthesis of Fmoc-Lys(Dnp) (24) (seeNote 2)

1. Dissolve fluoren-9-ylmethoxycarbonyl (Fmoc) *N*-hydroxysuccinimide ester (1.89 g; 5.60 mmol) in 30 mL of dimethoxyethane and store at 4 °C.
2. Dissolve Lys(Dnp) (1.63 g; 4.67 mmol) in 10 mL of 10% aqueous Na2CO3 and add slowly to the dimethoxyethane solution. Allow the reaction to proceed for 2 h at 4 °C or overnight at room temperature.
3. Filter the solution and acidify the filtrate to pH ~3 with concentrated HCl.
4. Remove the dimethoxyethane by heating the solution under reduced pressure.
5. Extract the solution with ethyl acetate, and reduce the ethyl acetate layer by heating under reduced pressure to an oil.
6. The oil [Fmoc-Lys(Dnp)] can be used without further purification. Alternatively (94), dissolve the oil in $CHCl_3$ (30 mL) and apply to a column (2 cm × 7 cm) of silica gel 60 (Merck 9385). Elute the product with 60 mL of $CHCl_3$–methanol–acetic acid (9.25:0.5:0.25, v/v).
7. Remove the solvents *in vacuo* leaving an oil that crystallizes upon tritration with light petroleum. Fmoc-Lys(Dnp) can be recrystallized as a dicyclohexylamine salt.

3.1.2. Fmoc-Lys(Dnp)-Gly-4-hydroxymethylphenoxy (HMP) resin (24)

1. Dissolve Fmoc-Gly (5.77 g; 19.4 mmol), 1-hydroxybenzotriazole (HOBt) (2.97 g; 19.4 mmol), and 4-dimethylaminopyridine (DMAP) (0.237 g; 1.94 mmol) in 100 mL DCM–*N*,*N*-dimethylformamide (DMF) (1:1) and add to 5.0 g HMP resin (4.85 mmol).
2. Add *N*,*N*′-diisopropylcarbodiimide (DIPCDI) (3.04 mL; 19.4 mmol), and allow esterification to proceed for 3 h.
3. Add an additional 200 mL of DMF, and allow the reaction to continue for 3.5 h.
4. Wash the resin with DMF and DCM and store under vacuum overnight. Fulvene-piperidine analysis (95) gives a substitution level of 0.45 mmol/g for Fmoc-Gly-HMP resin.

[2]The synthesis of other Fmoc-derivatives, such as Fmoc-Tyr(NO_2) and Fmoc-Abz, follows methods similar to that described here (111). Many of these derivatives are commercially available [such as Fmoc-Lys(Mca)].

5. Deprotect 3.0 g of Fmoc-Gly-HMP resin (1.36 mmol) by treating with 50 mL piperidine–DMF (1:4) for 0.5 h and washing 3 times with DMF.
6. Dissolve Fmoc-Lys(Dnp) (0.232 g; 4.07 mmol) and HOBt (0.623 g; 4.07 mmol) in 50 mL of DCM–DMF (1:1) and add to the resin.
7. Add DIPCDI (0.637 mL; 4.07 mmol) and allow coupling to proceed for 3.5 h.
8. Wash the resultant Fmoc-Lys(Dnp)-Gly-HMP resin with DMF and DCM and store under vacuum.

The attempted synthesis of peptide analogues using *stored* Fmoc-Lys(Dnp)-Gly-HMP resin produces peptides which, by FABMS analysis, are missing the *C*-terminal Lys(Dnp)-Gly (-352.1 Da) (24). Thus, the initial base treatment of the stored Fmoc-Lys(Dnp)-Gly-HMP resin causes diketopiperazine formation (95). The diketopiperazine side-reaction can be avoided by incorporating Fmoc-Lys(Dnp) onto an amide-producing peptide-resin linker 4-(2′,4′-dimethoxyphenylaminomethyl)phenoxy (DMPAMP).

3.1.3. Fmoc-Lys(Dnp)-DMPAMP resin (24)

1. Deprotect 2.0 g of Fmoc-DMPAMP resin (0.86 mmol) by treatment with 20 mL piperidine–DMF (1:1) for 0.5 h and washing 3 times with DMF.
2. Dissolve Fmoc-Lys(Dnp) (0.2 g; 3.5 mmol) and HOBt (0.536 g; 3.5 mmol) in 20 mL of DCM–DMF (1:1) and add to the resin.
3. Add DIPCDI (0.548 mL; 3.5 mmol) and allow coupling to proceed for 4.5 h.
4. Wash the resultant Fmoc-Lys(Dnp)-DMPAMP resin with DMF and DCM and store under vacuum.

3.1.4. N^2-Fmoc-N^3-Dnp-2,3-diaminopropionic acid (Fmoc-Dpa) (22)

1. Dissolve Fmoc-Asn (1.42 g, 4.0 mmol) in 10 mL DMF.
2. Add H_2O (2.0 mL), pyridine (0.75 mL, 8.0 mmol) and [bis(trifluoroacetoxy)iodo]benzene (2.60 g, 6.0 mmol), and stir the mixture at 20 °C overnight.
3. Remove the solvents are at 50 °C *in vacuo* and dissolve the remaining oil in H_2O (100 mL) and concentrated HCl (10 mL).
4. Extract the solution twice with diethyl ether (50 mL each) and bring to pH 7 with solid Na_2CO_3.
5. Add $NaHCO_3$ (0.67 g, 8.0 mmol), ethanol (100 mL) and 1-fluoro-2,4-dinitrobenzene (0.5 mL, 4.0 mmol), and stir the mixture at 20 °C for 2 h.
6. Remove ethanol at 40 °C *in vacuo* and bring the aqueous solution to pH 1 with HCl.
7. Recrystallize the crude product (1.59 g, 81% yield) from hot ethanol/H_2O.

3.1.5. Fmoc-L-Amp, Method 1 (96)

1. Suspend (2*R*)-Borane-10,2-sultam glycinate (Oxford Asymmetry, Abingdon) (1.89 g, 5.0 mmol), 4-bromomethyl-7-methoxycoumarin (Fluka) (1.62 g, 6.0 mmol), NBu_4HSO_4 (2.04 g, 6.0 mmol) and $LiOH•H_2O$ (0.84 g, 20.0 mmol) in DCM (45 mL) and H_2O (7.5 mL), and stir vigorously for 18 h at 4 °C.
2. Filter the mixture, dilute with DCM (50 mL), extract twice with H_2O (50 mL each) and dry over $MgSO_4$.
3. Evaporate the DCM, and isolate the product, a coumarin-sultam adduct, as a yellow foam by flash chromatography on SiO_2 eluted with $CHCl_3$–methanol (98:2).
4. Dissolve the coumarin-sultam adduct in tetrahydrofuran (100 mL) and stir with 1 M HCl (50 mL) at room temperature. After 18 h, hydrolysis of the bis(methylthio)methylidene group is complete and aminoacyl-sultam is formed.
5. Filter the reaction mixture and evaporate solvents.
6. Add H_2O (50 mL) and evaporate. Wash the residual solid twice with diethyl ether (30 mL each).
7. Dissolve the aminoacyl-sultam in tetrahydrofuran (100 mL) and stir with 0.4 M $LiOH•H_2O$ in H_2O at room temperature for 1 h.
8. Evaporate the solvents to 50 mL, add H_2O (50 mL) and extract the solution twice with DCM (50 mL each).
9. Filter the aqueous phase, reduce in volume to 60 mL, and neutralize with 12 M HCl.
10. Stir the precipitate of L-2-amino-3-(7-methoxy-4-coumaryl)-propionic acid (L-Amp) at 0 °C for 24 h and recover by filtration (1.06 g, 81% yield).
11. Prepare Fmoc-L-Amp by standard methods using Fmoc *N*-hydroxysuccinimide ester (37).

3.1.6. Fmoc-L-Amp, Method 2 (97)

1. Dissolve benzyl (2*R*,3*S*)-(–)-6-oxo-2,3-diphenyl-4-morpholine-carboxylate (Aldrich) (216 mg, 0.56 mmol) and 4-bromomethyl-7-methoxycoumarin (Fluka) (100 mg, 0.37 mmol) in tetrahydrofuran (20 mL) and cool to the temperature of dry ice–acetone (−78 °C). Add sodium bis(trimethylsilyl)amide (280 μL, 2 M solution in tetrahydrofuran, 0.56 mmol) dropwise via a syringe and stir the solution for 2 h.
2. Warm up the solution from above to room temperature and then pour it onto DCM (20 mL). Wash the solution with H_2O and brine, dry over anhydrous Na_2SO_4, filter, and concentrate.
3. Purify the yellowish solid by preparative thin-layer chromatography (eluted with DCM–ethyl acetate, 20:1) to yield an oxazinone intermediate as a white powder.
4. Dissolve the oxazinone intermediate in tetrahydrofuran and hydrogenate the solution for 24 h in the presence of 0.5 equiv. of $PtCl_2$ catalyst.

5. Add methanol to dissolve the precipitated amino acid and filter the solution through Celite. Concentrate the solution.
6. Triturate the precipitate with diethyl ether and collect the product [L-2-amino-3-(7-methoxy-4-coumaryl)-propionic acid (L-Amp)] by centrifugation.
7. Prepare Fmoc-L-Amp by standard methods using Fmoc *N*-hydroxysuccinimide ester (60).

3.1.7. Boc-Glu(Edans)-OH (98)

1. Mix Edans (Sigma) (300 mg, 1.13 mmol), Boc-Glu-OBzl (381 mg, 1.13 mmol), and benzotriazolyl *N*-oxytrisdimethylaminophosphonium hexafluorophosphate (BOP) (489 mg, 1.13 mmol) with 10 mL of dry DMF.
2. Add *N*,*N*-diisopropylethylamine (DIEA) (392 μL, 2.25 mmol) to the suspension, and stir the mixture at room temperature under a nitrogen atmosphere for 4 h, yielding a clear solution.
3. Remove DMF by rotary evaporation under reduced pressure, and purify the crude residue twice by flash chromatography, once in methanol (saturated with NH_3)–DCM (1:4), followed by methanol–acetic acid–ethyl acetate (6:1:43) to yield 486 mg (71%) of Boc-Glu(Edans)-OBzl as a pale gold solid after lyophilization from H_2O.
4. Dissolve the product (~120 mg) in 30 mL methanol in a Parr bottle. Add Pd/C (10%; 33 mg) and shake the reaction in a Parr apparatus under an H_2 atmosphere at 40 psi for 8 h.
5. Remove the catalyst by filtration, and remove the solvent by evaporation under reduced pressure to yield Boc-Glu(Edans)-OH.

3.1.8. Fmoc-Glu(Edans)-OH (99)

1. Mix Edans (Sigma) (1.07 g, 4 mmol), Fmoc-Glu-O*t*Bu (1.70 g, 4 mmol) and BOP (1.77 g, 4 mmol) with 10 mL of dry DMF.
2. Add DIEA (2 mL, 12 mmol) to the suspension and stir the mixture at room temperature under a nitrogen atmosphere for 2 h, yielding a clear solution.
3. Add DCM (50 mL) and 0.5 M $KHSO_4$ (50 mL), and wash the organic layer with 0.1 M $KHSO_4$ and H_2O, dry over $MgSO_4$ and evaporate *in vacuo* to yield 2.8 g of an oil.
4. Add glacial acetic acid (10 mL) and 36% HCl (1 mL), and stir the solution for 1 h.
5. Evaporate the solvents *in vacuo*, and remove traces of acetic acid by co-evaporation with DMF (2 × 10 mL).
6. Purify the product, in 45% yield, by C_{18} RP-HPLC.

3.2. Methods For the Synthesis of Fluorogenic Substrates

3.2.1 Substrates containing Dnp, Trp, Edans, Mca, Dabcyl, Abz, and/or Tyr(NO_2)

1. Incorporation of individual amino acids has typically been by Fmoc solid-phase methodology (22, 24, 26, 99), although the Boc strategy has been utilized for substrates containing Dnp and Trp (20). It is recommended that for Trp-containing substrates the Fmoc-Trp(Boc) derivative be utilized (100). Dnp is incorporated at the *N*-terminus of the peptide by using the appropriate, commercially available Dnp-amino acid derivative for the final coupling (25).
2. Couple Fmoc-Glu(Edans)-OH using a 10-fold excess with benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP)/*N*-methylmorpholine (NMM) for 3 h (99).
3. Peptide-resins may be analyzed prior to *N*-terminal acylation by Edman degradation analysis to evaluate the efficiency of assembly (24).
4. Acylate the *N*-termini of peptide-resins with 7-methoxycoumarin-4-acetic acid using standard synthesis cycles with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HBTU) and HOBt and 2–4 h coupling times (24) or multiple couplings with PyBOP and HOBt (22). *N*-terminal acylation with Dabcyl is performed using 10 equiv. *p*-(*p*-dimethylaminophenylazo)benzoic acid (Sigma), 10 equiv. PyBOP, and 20 equiv. NMM in 250 μL *N*-methylpyrrolidone (NMP) for 3 h (99) (seenote 3).

3.2.2. Substrates containing Lys(Nma)—To create a fluorogenic substrate containing Lys(Nma), the peptide is assembled by Boc chemistry using a Boc-Lys(Fmoc) derivative (25).

1. Treat the peptide-resin with thiophenol (2 mL) in NMP (15 mL) for 1 h to remove Dnp side-chain protection from His if present (note: this treatment will *not* remove an N^{α}-Dnp group).
2. Wash the peptide-resin with DCM (6 × 20 mL) and remove Fmoc side-chain protection with two treatments of piperidine (2 mL) in NMP (15 mL) for 20 min each.
3. React the resin with succinimidyl *N*-methylanthranilate (Molecular Probes, Eugene, OR) (1 mmol) in NMP (15 mL) with DIEA (1.1 mmol) for 16 h.
4. Wash the resin with DCM (6 × 20 mL) and dry.

   This substrate could also be assembled using orthogonal side-chain (ε-amino) protection for either Boc- and Fmoc-Lys provided by the palladium-sensitive allyloxycarbonyl (Aloc) group (101, 102). In addition, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) side-chain protection of Lys during Fmoc

[3]One can also prepare Dabcyl-N-hydroxysuccinimide ester (Dabcyl-NHS) for incorporation onto the ε-amino group of Fmoc-Lys, if the Dabcyl group is needed within the peptide sequence (112).

chemistry allows for selective deprotection with 2% hydrazine in DMF (103). Partial loss of Dde moiety had been noted during the synthesis of long sequences (104), compromising the purity of the final product. Furthermore, Dde has also been reported to undergo intramolecular $N \rightarrow N$ migration, leading to scrambling of its position within the peptide chain. In order to avoid both side reactions, 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl (ivDde) has been introduced (104). The ivDde group is cleaved under the same conditions as Dde group. Another selectively removable side-chain protecting group for Lys is 4-methyltrityl (Mtt), which is labile to 1% TFA–triisopropylsilane in DCM (105).

5. Cleave peptides from the resin and deprotect the side-chains by treatment with HF plus appropriate scavengers (20, 25) or TFA plus appropriate scavengers (100, 106).
6. Purify peptides by preparative C18 RP-HPLC (22, 24, 25).
7. Characterize peptides by electrospray mass spectrometry (24), FABMS (24, 25) or matrix-assisted laser desorption/ionization mass spectrometry (26, 99).

3.2.3. Substrates containing LY and Ctmr—When the combination of LY and Ctmr are used for a substrate, both the fluorophore and quencher are incorporated after peptide synthesis and purification (27, 28). The peptide is synthesized with an *N*-terminal Ser, which is converted by periodate oxidation to an *N*α-glyoxylyl group as follows.

1. Dissolve peptide (7.7 μmol) in 0.25 mL of 25 mM sodium phosphate, pH 7.0.
2. Titrate the resulting solution with 1 μL drops of 1 M NaOH until the pH is near 7.
3. Treat the solution with 0.25 mL of 40 mM M $NaIO_4$ dissolved in 25 mM sodium phosphate, pH 7.0 (final pH of the periodate solution, pH 6.7, not further adjusted), to create a solution at 22 °C containing 20 mM $NaIO_4$ and 15 mM peptide. After 15 min the peptide should be converted to the N^{α}-glyoxylyl form.
4. Treat the solution with 1 mL of 30 mM Lucifer Yellow CH (Molecular Probes, Eugene, OR) in 0.1 M sodium acetate, pH 4.5, and incubate the resulting 1.5 mL of reaction mixture at 22 °C for 2 h.
5. The glyoxylyl group forms a hydrazone with the carbohydrazide LY. Isolate the product by C_{18} RP-HPLC.
6. React the free N^{ε}-amino group of Lys with 5-carboxytetramethylrhodamine succinimidyl ester as follows. Redissolve the LY-peptide (~11 mg) in 2.25 mL of 0.1 M $NaHCO_3$ and treat with 0.75 mL of 5-carboxytetramethylrhodamine succinimidyl ester (Molecular Probes, Eugene, OR) prepared as a 20 mM suspension in acetonitrile.
7. After 1 h at 22 °C, add 0.15 mL of the suspension of 5-carboxytetramethylrhodamine succinimidyl ester to drive the reaction toward completion. Purify the LY-peptide-Ctmr product by C_{18} RP-HPLC.

3.2.4. Substrates containing Cy3 and Cy5Q—When the combination of Cy3 and Cy5Q are used for a substrate, the Cy3 fluorophore is added to the peptide “on-resin” while the Cy5Q quencher is incorporated after peptide synthesis and purification (36).

1. Assemble acetyl-Arg-Pro-Lys(Mtt)-Pro-Val-Glu-Nva-Trp-Arg-Lys(Boc)-DMPAMP amide resin by standard Fmoc solid-phase peptide synthesis.
2. To remove the 4-methyltrityl (Mtt) group, suspend the peptide-resin in TFA–TIS–DCM (1:5:94) and shake for 3 min. Wash the peptide-resin twice with DCM.
3. Cy3 mono acid (Amersham Biosciences) (1 equiv.) is dissolved in a solution of 7-azobenzotriazolyoxytris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP, 2 equiv.) in NMP. Anhydrous DIEA (5 equiv.) is added, and the dye solution is allowed to stand for 3–5 min before addition to the peptide-resin.
4. Incubate the dye solution and the peptide-resin in the dark at 22–25 °C, and wash the peptide-resin with NMP and DCM.
5. Acylate unlabeled sites by treatment of the peptide-resin with 0.5 M acetic anhydride, 0.125 M DIEA, and 15 mM HOBt in NMP for 30 min at 22–25 °C. Wash the peptide-resin with NMP and DCM.
6. Cleave the peptide from the resin using water–TIS–TFA (2.5:2.5:95) in the dark at 22–25 °C for 4 h. Precipitate the crude product with a 10-fold excess of cold diethyl ether and centrifuge at 2000 g for 5 min. Decant the ether off.
7. Wash the crude product twice with diethyl ether. Purify the peptide by RP-HPLC using a Jupiter C_{18} column (Phenomenex) with a linear gradient and eluents of A = 0.1% TFA/water and B = 0.1% TFA/acetonitrile. Lyophilize the peptide.
8. Incubate the Cy3-labeled peptide with Cy5Q NHS ester (Amersham Biosciences) (1.1 equiv.) in anhydrous dimethylformamide and DIEA (4% by volume) in the dark at 22–25 °C for 60 min.
9. Isolate the dual-labeled peptide by ion-exchange chromatography on a HiTrap Q Sepharose 5-mL column (Amersham Biosciences), where eluent A = 0.1 M borate buffer (pH 9.0) and eluent B = 1 M NaCl. Desalt the peptide using a Sep Pak Plus C18 cartridge (Waters) and lyophilize.
10. Characterize the peptide by LC-MS and spectroscopic analysis at λ = 550 and 650 nm.

3.3. Protocols for Assaying Matrix Metalloproteinases Using Fluorogenic Substrates (seenote 4)

3.3.1. Dnp-Pro-Leu-Gly~Leu-Trp-Ala-D-Arg-NH_2 (20)

1. Prepare the substrate as a stock solution in dimethylsulfoxide (DMSO) and determine the concentration spectrophotometrically using ε_{372} = 16,000 $M^{-1}cm^{-1}$ (seenote 5).
2. Perform assays in assay buffer A, at 37 °C using peptide concentrations ranging from 2.5 – 40 μM.
3. Add enzyme, and determine the initial rate of substrate hydrolysis at λexcit = 280 nm and λemiss = 346 nm.
4. Calibrate the fluorimeter by adding aliquots of a standard solution of Trp directly to the reaction mixture at the conclusion of each run. Fluorescent intensities are not corrected for the inner filter effect as the maximum correction factor is less than 8% at the highest substrate concentration.

3.3.2. Dnp-Arg-Pro-Lys-Pro-Leu-Ala~Nva-Trp-NH_2 and Dnp-Arg-Pro-Lys-Pro-Leu-Ala~Phe-Trp-NH_2 (21)

1. Prepare substrates as stock solutions in DMSO.
2. Perform assays using 2.96 mL of assay buffer B and 20 μL substrate in a 3 mL cuvette at 25 °C.
3. Initiate the reaction by the addition of 20 μL of enzyme solution.
4. Monitor reaction progress at λex = 290 nm and λem = 340 nm. Values of k_{cat}/K_M are calculated from first-order progress curves for fluorescence increase with $[S]_O = 2\ \mu M \ll K_M$.

3.3.3. NFF-1, NFF-2, and NFF-3 (24)

1. Prepare substrates as 10 mM stock solutions in DMSO.
2. Perform fluorescent assays at λex = 325 nm and λem = 393 nm.
3. Run initial total hydrolysis assays at a substrate concentration of 5 μM to avoid filtering effects. The change in fluorescence at this concentration is 110 based on total hydrolysis of 1 μM substrate.

[4]The fluorophores exhibiting greater quantum yields allow for the most sensitive assays, provided that quenching is reasonably efficient. On this basis, substrates using Mca are preferred over Trp, Nma, or Edans (18). Trp is the most problematic fluorophore, primarily because assays could suffer from high background due to MMP or other protein internal Trp residues.

[5]Solubility in aqueous buffer is a problem for many fluorogenic substrates due to the hydrophobic nature of fluorescent donors and acceptors. The solubility of the Dabcyl-Gaba-Pro-Gln-Gly~Leu-Glu(Edans)-Ala-Lys-NH_2 substrate in assay buffer F is 80 μM, while the solubilities of Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH2 and Dnp-Pro-Cha-Gly~Cys(CH3)-His-Ala-Lys(Nma)-NH2 in the same buffer are 4.3 and 4.6 μM, respectively (26). NFF-3 is soluble up to a concentration of 250 μM using 2.5% DMSO in water (24), which appears to be better than the solubility of Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH2 (22). The modified Mca-Lys-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH2 substrate can be prepared as a 20 mM stock solution in water at stored at −20°C (51).

4. Incubate 100 μL of various concentrations of a substrate with 10 μL of an enzyme solution (2 – 20 nM) in assay buffer C at 37 °C (MMP-3 activity at pH 6.0 is measured in assay buffer D, instead of Tris-HCl buffer).
5. Stop the reaction by addition of 900 μL of 3% (v/v) glacial acetic acid.

The amount of substrate hydrolysis is calculated based on the fluorescence values of the Mca-Arg-Pro-Lys-Pro-Gln standard solution after subtraction of the reaction blank value (stopping solution added before the enzyme). Individual kinetic parameters (kcat and KM) are determined over a substrate concentration range of 2.5 – 75 μM and calculated by double reciprocal plots or non-linear regression analysis.

3.3.4. Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH2 (18, 22)

1. Prepare substrate as a stock solution in DMSO and determine the concentration spectrophotometrically using ε_{410} = 7,500 $M^{-1}cm^{-1}$.
2. Perform fluorescent assays at at λex = 328 nm and λem = 393 nm.
3. Zero the fluorimeter with substrate in assay buffer E and then calibrate with Mca-Pro-Leu so that the full-scale deflection corresponds to between 2 and 10% hydrolysis of the substrate.

   For each MMP, the initial rate of substrate cleavage, measured over 10 – 15 min, was proportional to substrate concentration in the range of 1–8 μM. A concentration of 1.6 μM is used to determine k_{cat}/K_M values. Absorptive quenching at higher substrate concentrations can occur, and it is thus important to calibrate at each substrate concentration. Inner filter effect correction factors can be applied if needed (51, 107) (see alsonote 6).
4. To determine the extent of quenching, zero the fluorimeter with buffer in the cuvette and measure the fluorescence of substrate solutions of increasing concentration.
5. Plot the values versus substrate concentration and extrapolate the initial linear portion of the curve. The difference between the extrapolated and experimental points at any concentration shows the extent of quenching.

Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2 has also been used for flow injection analysis of MMP-7 activity and inhibitors (108).

3.3.5. Dabcyl-Gaba-Pro-Gln-Gly~Leu-Glu(Edans)-Ala-Lys-NH_2 (26)

1. Perform fluorescent assays at λex = 340 nm and λem = 485 nm.
2. Measure purified MMP activity at enzyme concentrations of 1.5 – 25 nM with 1.8 μM substrate in assay buffer F at 37 °C. Crude MMP activity is measured using 5 μM substrate in a total volume of 150 μL assay buffer at 37 °C.

[6]The inner filter effect (IFE) is often observed when using fluorogenic substrates at high concentrations. Decreases in fluorescence due to IFE exceed 10% once the sum of the absorbances at excitation and emission wavelengths is greater than 0.08 (113). Correction factors can be utilized based on IFEs (107). Alternatively, fitting of reaction progress curves can be used to obtain accurate kinetics even when IFE has occurred (113).

Dabcyl-Gaba-Pro-Gln-Gly~Leu-Glu(Edans)-Ala-Lys-NH_2 has been used to monitor MMP-3 activity in rheumatoid synovial fluid (109).

3.3.6. Dnp-Pro-Cha-Gly~Cys(CH3)-His-Ala-Lys(Nma)-NH2 (25)

1. Prepare substrate as a 5 mM stock solution in DMSO with the concentration determined spectrophotometrically using ε_{372} = 16,000 $M^{-1}cm^{-1}$.
2. Perform fluorescent assays at λex = 340 nm and λem = 440 nm.
3. Determine the k_{cat}/K_M values at a substrate concentration of 0.5 μM in a quartz cuvette containing 3 mL of assay buffer G at 23 °C.
4. For microtiter plates, conduct assays in a total volume of 0.3 mL assay buffer containing 3 nM MMP-1 in each well of a black 96-well plate.
5. Initiate assays by substrate addition (10 μM final concentration) and measure the product formation at λex = 365 nm and λem = 450 nm after 40 – 60 min.

3.3.7. LY-Gly-Pro-Leu-Gly~Leu-Arg-Ala-Lys(Ctmr) (27)

1. Measure MMP activity at room temperature by monitoring the change in fluorescence of a sample containing various amounts of substrate (0.1 – 4 μM) and enzyme dissolved in assay buffer H.
2. Excite the fluorescent substrate at 430 nm (10 nm slit) and read emission at 530 nm (5 nm slit).

Filtering effects of LY and fluorescence of Ctmr may need to be considered at higher substrate concentrations (27, 28) (seenote 6).

3.3.8. fTHP-15 [$(Gly\text{-}Pro\text{-}Hyp)_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly~Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-$(Gly\text{-}Pro\text{-}Hyp)_5$-$NH_2$] for HTS (83) (seenote 7)

1. All experiments are performed in 1536-well white microtiter plates (Greiner Bio-One, Monroe, NC). All reagents are dispensed at ambient temperature. To begin the assay, 2.5 μL of 8 μM fTHP-15 in EAB is added to the wells using a FRD™ IB Workstation (Aurora Discovery, Carlsbad, CA).
2. Twenty nL of DMSO–water (3:1) containing the control compounds, test compounds, or no compounds is dispensed using a 1536-head Pintool system (GNF Systems, San Diego, CA). Final assay concentrations for inhibition control

[7]It has been noted that fTHPs containing Lys(Mca), while having high quantum yields and relative ease of assembly, may not be optimal, as the side-chain of Lys results in (a) the Mca group being distant from the peptide backbone and potential steric interference with the enzyme and (b) increased overall hydrophobicity of the substrate (60). One study compared the MMP activities of an fTHP sequence containing one of four different fluorophores: L-2-amino-3-(7-methoxy-4-coumaryl)propionic acid (L-Amp); D-2-amino-3-(7-methoxy-4-coumaryl)propionic acid (D-Amp); L-2-amino-3-(6,7-dimethoxy-4-coumaryl)propionic acid [L-Adp, also referred to as [6,7-dimethoxy-4-coumaryl)alanine (Dca) (120)]; and D-2-amino-3-(6,7-dimethoxy-4-coumaryl)propionic acid (D-Adp)] (60). The shorter side-chain of Amp or Adp was better tolerated by MMP-1 and MMP-2 (60). Adp may well be the fluorophore of choice for fTHPs, as (a) fTHPs incorporating Adp were obtained in significantly higher yields than the Amp-containing fTHPs, (b) Adp has a larger Stokes shift than either Amp or Lys(Mca), and thus has less chance of self-quenching, (c) Adp has a relatively high quantum yield, (d) the Adp/Dnp pair is compatible with multi-well plate reader formats, and (e) MMPs better tolerate Adp compared with Lys(Mca) (60). The synthesis of Adp is very similar to that of Amp, Method 2, except that 4-bromomethyl-6,7-dimethoxycoumarin (Aldrich) is used instead of 4-bromomethyl-7-methoxycoumarin (114).

compounds are 80 nM of MMP-13 inhibitor for 50% inhibition control wells and 8 μM of MMP-13 inhibitor for 100% inhibition (positive) control wells. Test compounds are present in the assay at a final nominal concentration of 4 μM. The DMSO concentration in the assay is 0.3%.

3. Reactions are initiated by addition of 2.5 μL of 2.66 nM MMP-13 in EAB.
4. After 4 h of incubation at 25 °C the reaction is stopped by addition of 5 μL of 50 mM EDTA.
5. Plates are incubated for 10 min at 25 °C and emission fluorescence is read on the Viewlux (Perkin-Elmer, Turku, Finland) microplate reader (λ_{ex} = 325 nm, λ_{em} = 450 nm).
6. To determine dose-dependent inhibition, each compound is assayed in triplicate, using 10 point, 1:3 serial dilutions starting at a nominal test concentration of 40 μM.
7. From the raw fluorescence intensity values (RFU_{450}), the percent inhibition for each well is calculated as follows:

$$\text{Percent inhibition} = 100 \times ([\text{RFU}_{450}\ \text{test compound}] - [\text{median RFU}_{450}\ \text{negative control}]) / ([\text{median RFU}_{450}\ \text{positive contr}$$

where “test compound” is the well containing test compound, “positive control” is defined as test wells containing 8 μM MMP-13 inhibitor, and “negative control” is defined as test wells containing DMSO only.

8. To determine IC_{50} values for each compound, percentage inhibitions are plotted against compound concentration. A four parameter equation describing a sigmoidal dose-response curve is then fitted with adjustable baseline using Assay Explorer software (MDL Information Systems, San Ramon, CA). IC_{50} values are generated from fitted curves by solving for the X intercept at the 50% inhibition level of the Y intercept. In cases where the highest concentration tested (i.e., 40 μM) does not result in greater than 50% inhibition, the IC_{50} is determined manually as greater than 40 μM.

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Table 1

HYDROLYSIS OF FLUOROGENIC SUBSTRATES BY MMPS AND RELATED METALLOPROTEINASES

Substrate	k_{cat}[a] (sec^{-1})	K_M[a] (μM)	k_{cat}/K_M[a] ($sec^{-1}M^{-1}$)	Reference
MMP-1				
Dnp-Pro-Gln-Gly~Ile-Ala-Gly-Trp	ND	ND	230[b]	[Netzel-Arnett, 1991 #1307]
Dnp-Pro-Leu-Gly~Leu-Trp-Ala-D-Arg-NH_2	ND	ND	827	[Murphy, 1992 #130]
Dnp-Pro-Leu-Gly~Leu-Trp-Ala-D-Arg-NH_2	ND	ND	830	[Knight, 1992 #1292]
Mca-Arg-Pro-Lys-Pro-Tyr-Ala~Nva-Trp-Met-Lys(Dnp)-NH_2 (NFF-2)	0.15	142	1,060	[Nagase, 1994 #813]
Dnp-Pro-Leu-Gly~Leu-Trp-Ala-D-Arg-NH_2	0.011[c]	7.1[c]	1,500[c]	[Stack, 1989 #1290]
Mca-Pro-Lys-Pro-Gln-Gln~Phe-Phe-Gly~Leu-Lys(Dnp)-Gly (NFF-1)	0.04	16.6	2,510	[Nagase, 1994 #813]
Dnp-Pro-Leu-Ala~Tyr-Trp-Ala-Arg	0.13[b]	50[b]	2,700[b]	[Netzel-Arnett, 1991 #1307]
Dnp-Pro-Leu-Ala~Leu-Trp-Ala-Arg	1.2[b]	130[b]	9,400[b]	[Netzel-Arnett, 1991 #1307]
Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	12,100[d]	[Knäuper, 1996 #1324]
Mca-Pro-Cha-Gly~Nva-His-Ala-Dpa-NH_2	ND	ND	14,700[d]	[Knäuper, 1996 #1324]
Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	14,800	[Knight, 1992 #1292]
Mca-Lys-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	2.5[d]	27.5[d]	92,200[d]	[Neumann, 2004 #2671]
Dabcyl-Gaba-Pro-Gln-Gly~Leu-Glu(Edans)-Ala-Lys-NH_2	ND	ND	21,000	[Beekman, 1996 #902]
LY-Gly-Pro-Leu-Gly~Leu-Arg-Ala-Lys(Ctmr)	ND	3.9	ND	[Geoghegan, 1993 #1294]
Dns-Pro-Leu-Ala~Leu-Trp-Ala-Arg-NH_2	0.417[h]	145[h]	7,300[h]	[Holtz, 1999 #3301]
Dns-Pro-Leu-Ala~Cys(Mob)-Trp-Ala-Arg-NH_2	0.150[h]	17	8,800[h]	[Holtz, 1999 #3301]
Dnp-Pro-Cha-Gly~Cys(Me)-His-Ala-Lys(Nma)-NH_2	ND	ND	30,000	[Mohan, 2002 #2459]
Dabcyl-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(Fam)-NH_2	ND	ND	76,000	[Moss, 2007 #3292]
Dabcyl-Leu-Ala-Gln-Ala-Homophe-Arg-Ser-Lys(Fam)-NH_2	ND	ND	28,000	[Moss, 2007 #3292]
fTHP-4; $(Gly\text{-}Pro\text{-}Hyp)_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly~Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-$(Gly\text{-}Pro\text{-}Hyp)_5$-$NH_2$	0.06[k]	15.2[k]	3,900[k]	[Minond, 2006 #2761]
MMP-1(Δ243–450)				
Dnp-Pro-Leu-Gly~Leu-Trp-Ala-D-Arg-NH_2	ND	ND	653	[Murphy, 1992 #130]
fTHP-4; $(Gly\text{-}Pro\text{-}Hyp)_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly~Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-$(Gly\text{-}Pro\text{-}Hyp)_5$-$NH_2$	0.02[k]	13.3[k]	1,500[k]	[Minond, 2006 #2761]

Substrate	k_{cat}[a] (sec^{-1})	K_M[a] (μM)	k_{cat}/K_M[a] ($sec^{-1}M^{-1}$)	Reference
MMP-1 (19 kDa)				
Dnp-Pro-Leu-Gly~Leu-Trp-Ala-D-Arg-NH_2	ND	ND	3,350[b]	[Bickett, 1993 #1296]
Dnp-Pro-Cha-Gly~Cys(Me)-His-Ala-Lys(Nma)-NH_2	ND	ND	13,000[b]	[Bickett, 1993 #1296]
MMP-1(1–242) + MMP-3(248–460)				
Dnp-Pro-Leu-Gly~Leu-Trp-Ala-D-Arg-NH_2	ND	ND	511	[Murphy, 1992 #130]
MMP-2				
Dnp-Pro-Gln-Gly~Ile-Ala-Gly-Trp	ND	ND	4,200[b]	[Netzel-Arnett, 1991 #1307]
Dnp-Pro-Leu-Gly~Met-Trp-Ser-Arg	0.069[b]	7.8[b]	8,900[b]	[Netzel-Arnett, 1991 #1307]
Mca-Pro-Lys-Pro-Gln-Gln~Phe-Phe-Gly-Leu-Lys(Dnp)-Gly (NFF-1)	1.07	100	10,800	[Nagase, 1994 #813]
Mca-Arg-Pro-Lys-Pro-Tyr-Ala~Nva-Trp-Met-Lys(Dnp)-NH_2 (NFF-2)	2.70	50	54,400	[Nagase, 1994 #813]
Dnp-Pro-Leu-Gly~Leu-Trp-Ala-D-Arg-NH_2	ND	ND	58,000[d]	[Knight, 1992 #1292]
Dnp-Pro-Leu-Gly~Leu-Trp-Ala-D-Arg-NH_2	0.32[c]	2.6[c]	122,000[c]	[Stack, 1989 #1290]
Dabcyl-Gaba-Pro-Gln-Gly~Leu-Glu(Edans)-Ala-Lys-NH_2	ND	ND	619,000	[Beekman, 1996 #902]
Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	629,000[d]	[Knight, 1992 #1292]
Mca-Lys-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	687,000[d]	[Neumann, 2004 #2671]
Dabcyl-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(Fam)-NH_2	ND	ND	29,000	[Moss, 2007 #3292]
Dabcyl-Leu-Ala-Gln-Ala-Homophe-Arg-Ser-Lys(Fam)-NH_2	ND	ND	320,000	[Moss, 2007 #3292]
Dabcyl-His-Gly-Asp-Gln-Met-Ala-Gln-Lys-Ser-Lys(Fam)-NH_2	ND	ND	2,400	[Moss, 2007 #3292]
α1(V)436–447 fTHP; (Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Pro-Gly~Val-Val-Gly-Glu-Lys(Dnp)-Gly-Glu-Gln-(Gly-Pro-Hyp)$_5$-NH_2	0.062[k]	4.4[k]	14,000[k]	[Lauer-Fields, 2003 #2028]
MMP-3				
Dnp-Pro-Gln-Gly~Ile-Ala-Gly-Trp	ND	ND	61[b]	[Netzel-Arnett, 1991 #1307]
Dnp-Pro-Leu-Gly~Leu-Trp-Ala-D-Arg-NH_2	ND	ND	2,200	[Murphy, 1992 #130]
Dnp-Pro-Leu-Gly~Leu-Trp-Ala-D-Arg-NH_2	ND	ND	2,200	[Knight, 1992 #1292]
Dnp-Pro-Tyr-Ala~Tyr-Trp-Met-Arg-NH_2	0.24[b]	100[b]	2,400[b]	[Netzel-Arnett, 1991 #1307]
Mca-Pro-Lys-Pro-Gln-Gln~Phe-Phe-Gly-Leu-Lys(Dnp)-Gly (NFF-1)	0.53	50	10,900	[Nagase, 1994 #813]
Dnp-Arg-Pro-Lys-Pro-Leu-Ala~Phe-Trp-NH_2	ND	ND	17,000[d]	[Niedzwiecki, 1992 #768]
Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	23,000	[Knight, 1992 #1292]

Substrate	k_{cat}[a] (sec^{-1})	K_M[a] (μM)	k_{cat}/K_M[a] ($sec^{-1}M^{-1}$)	Reference
Mca-Lys-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	20,400[d]	[Neumann, 2004 #2671]
Dabcyl-Gaba-Pro-Gln-Gly~Leu-Glu(Edans)-Ala-Lys-NH_2	ND	ND	40,000	[Beekman, 1996 #902]
Mca-Arg-Pro-Lys-Pro-Tyr-Ala~Nva-Trp-Met-Lys(Dnp)-NH_2 (NFF-2)	3.95	66	59,400	[Nagase, 1994 #813]
Dnp-Arg-Pro-Lys-Pro-Leu-Ala~Nva-Trp-NH_2	ND	ND	45,000[d]	[Niedzwiecki, 1992 #768]
Mca-Arg-Pro-Lys-Pro-Val-Glu~Nva-Trp-Arg-Lys(Dnp)-NH_2 (NFF-3)	1.31[d]	20[d]	65,700[d]	[Nagase, 1994 #813]
Mca-Arg-Pro-Lys-Pro-Val-Glu~Nva-Trp-Arg-Lys(Dnp)-NH_2 (NFF-3)	5.40	25	218,000	[Nagase, 1994 #813]
Mca-Arg-Pro-Lys-Pro-Val-Glu~Nva-Trp-Arg-Lys(Dnp)-NH_2 (NFF-3)	2.20[e]	7.5[e]	302,000[e]	[Nagase, 1994 #813]
Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	6,500[d]	[Park, 2000 #1692]
Mca-Pro-Lys-Pro-Leu-Ala-Leu-Dpa-Ala-Arg-NH_2	ND	ND	45,300[d]	[Park, 2000 #1692]
Mca-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys(Dnp)-NH_2	ND	ND	20,700[d]	[Park, 2000 #1692]
Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2	ND	ND	80,200[d]	[Park, 2000 #1692]
Mca-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Arg-NH_2	ND	ND	1,200[d]	[Park, 2000 #1692]
Dnp-Pro-Cha-Gly~Cys(Me)-His-Ala-Lys(Nma)-NH_2	ND	ND	500	[Mohan, 2002 #2459]
Dabcyl-Leu-Ala-Gln-Ala-Homophe-Arg-Ser-Lys(Fam)-NH_2	ND	ND	4,000	[Moss, 2007 #3292]
MMP-3(Δ248–460)				
Dnp-Pro-Leu-Gly~Leu-Trp-Ala-D-Arg-NH_2	ND	ND	2,390	[Murphy, 1992 #130]
MMP-3(1–233) + MMP-1(229–450)				
Dnp-Pro-Leu-Gly~Leu-Trp-Ala-D-Arg-NH_2	ND	ND	2,750	[Murphy, 1992 #130]
MMP-7				
Dnp-Pro-Leu-Gly~Leu-Trp-Ala-D-Arg-NH_2	ND	ND	11,700	[Knight, 1992 #1292]
Dnp-Pro-Leu-Gly~Leu-Trp-Ala-D-Arg-NH_2	ND	ND	13,000[f]	[Crabbe, 1992 #3294]
Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	169,000	[Knight, 1992 #1292]
Mca-Lys-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	295,000[d]	[Neumann, 2004 #2671]
Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	131,000[d]	[Park, 2000 #1692]
Mca-Pro-Lys-Pro-Leu-Ala-Leu-Dpa-Ala-Arg-NH_2	ND	ND	320,000[d]	[Park, 2000 #1692]
Mca-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys(Dnp)-NH_2	ND	ND	28,300[d]	[Park, 2000 #1692]
Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2	ND	ND	5,620[d]	[Park, 2000 #1692]

Substrate	k_{cat}[a] (sec^{-1})	K_M[a] (μM)	k_{cat}/K_M[a] ($sec^{-1}M^{-1}$)	Reference
Mca-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Arg-NH_2	ND	ND	3,420[d]	[Park, 2000 #1692]
Dns-Pro-Leu-Ala~Leu-Trp-Ala-Arg-NH_2	3.5[h]	114[h]	23,000[h]	[Holtz, 1999 #3301]
Dns-Pro-Leu-Ala~Cys(Mob)-Trp-Ala-Arg-NH_2	0.090[h]	20[h]	5,000[h]	[Holtz, 1999 #3301]
Dnp-Pro-Cha-Gly~Cys(Me)-His-Ala-Lys(Nma)-NH_2	ND	ND	10,000[i]	[Mohan, 2002 #2459]
MMP-8				
Dnp-Pro-Gln-Gly~Ile-Ala-Gly-Trp	21.0[b]	1100[b]	19,000[b]	[Netzel-Arnett, 1991 #1307]
Dnp-Pro-Leu-Ala~Leu-Trp-Ala-Arg	9.4[b]	50[b]	190,000[b]	[Netzel-Arnett, 1991 #1307]
Dnp-Pro-Leu-Ala~Tyr-Trp-Ala-Arg	3.1[b]	7.7[b]	400,000[b]	[Netzel-Arnett, 1991 #1307]
Mca-Lys-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	418,000[d]	[Neumann, 2004 #2671]
Dabcyl-Leu-Ala-Gln-Ala-Homophe-Arg-Ser-Lys(Fam)-NH_2	ND	ND	140,000	[Moss, 2007 #3292]
fTHP-4; $(Gly\text{-}Pro\text{-}Hyp)_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly~Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-$(Gly\text{-}Pro\text{-}Hyp)_5$-$NH_2$	0.035[k]	7.6[k]	4,500[k]	[Minond, 2006 #2761]
C_{10}-fTHP-4	0.089[k]	14.4[k]	6,200[k]	[Minond, 2006 #2761]
C_{16}-fTHP-4	0.016[k]	9.95[k]	1,600[k]	[Minond, 2006 #2761]
fTHP-9; $(Gly\text{-}Pro\text{-}Hyp)_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly~**Cys(Mob)**-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-$(Gly\text{-}Pro\text{-}Hyp)_5$-$NH_2$[l]	0.94[k]	5.4[k]	175,000[k]	[Minond, 2006 #2761]
C_{10}-fTHP-9	0.64[k]	8.8[k]	73,000[k]	[Minond, 2006 #2761]
C_{16}-fTHP-9	0.13[k]	9.6[k]	14,000[k]	[Minond, 2006 #2761]
fTHP-10; $(Gly\text{-}Pro\text{-}Hyp)_5$-Gly-Pro-Lys(Mca)-Gly-Pro-**Orn**-Gly~Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-$(Gly\text{-}Pro\text{-}Hyp)_5$-$NH_2$[l]	0.035[k]	8.15[k]	4,300[k]	[Minond, 2006 #2761]
fTHP-11; $(Gly\text{-}Pro\text{-}Hyp)_5$-Gly-Pro-Lys(Mca)-Gly-Pro-**Orn**-Gly~**Cys(Mob)**-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-$(Gly\text{-}Pro\text{-}Hyp)_5$-$NH_2$[l]	0.38[k]	19.7[k]	19,000[k]	[Minond, 2006 #2761]
C_{10}-fTHP-11	0.007[k]	3.60[k]	1,900[k]	[Minond, 2006 #2761]
C_{16}-fTHP-11	0.007[k]	3.50[k]	2,100[k]	[Minond, 2006 #2761]
C_6-fTHP-12; C_6-$(Gly\text{-}Pro\text{-}Hyp)_5$-Gly-Pro-Lys(Mca)-Gly-Pro-**Leu**-Gly~**Met**-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-$(Gly\text{-}Pro\text{-}Hyp)_5$-$NH_2$[l]	0.070[k]	14.5[k]	5,090[k]	[Minond, 2007 #2768]
fTHP-13; C_n-$(Gly\text{-}Pro\text{-}Hyp)_5$-Gly-Pro-Lys(Mca)-Gly-Pro-**Val**-**Asn**~**Phe**-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-$(Gly\text{-}Pro\text{-}Hyp)_5$-$NH_2$[l]	0.028[k]	9.30[k]	3,010[k]	[Minond, 2007 #2768]
C_6-fTHP-13	0.020[k]	3.90[k]	3,870[k]	[Minond, 2007 #2768]
MMP-8 (Met80 or Leu81 N-terminus)				
Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	135,000[e]	[Knäuper, 1996 #1324]

Substrate	k_{cat}[a] (sec^{-1})	K_M[a] (μM)	k_{cat}/K_M[a] ($sec^{-1}M^{-1}$)	Reference
Mca-Pro-Cha-Gly~Nva-His-Ala-Dpa-NH_2	ND	ND	166,000[e]	[Knäuper, 1996 #1324]
MMP-8 (Phe79 N-terminus)				
Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	193,000[d]	[Knäuper, 1996 #1324]
Mca-Pro-Cha-Gly~Nva-His-Ala-Dpa-NH_2	ND	ND	252,000[d]	[Knäuper, 1996 #1324]
MMP-9				
Dnp-Pro-Gln-Gly~Ile-Ala-Gly-Trp	ND	ND	1,200[b]	[Netzel-Arnett, 1991 #1307]
Mca-Arg-Pro-Lys-Pro-Val-Glu~Nva-Trp-Arg-Lys(Dnp)-NH_2 (NFF-3)	0.282	28	10,100	[Nagase, 1994 #813]
Dnp-Pro-Leu-Gly~Met-Trp-Ser-Arg	0.069[b]	5.2[b]	13,000[b]	[Netzel-Arnett, 1991 #1307]
Mca-Arg-Pro-Lys-Pro-Tyr-Ala~Nva-Trp-Met-Lys(Dnp)-NH_2 (NFF-2)	1.38	25	55,300	[Nagase, 1994 #813]
Dnp-Pro-Leu-Gly~Leu-Trp-Ala-D-Arg-NH_2	ND	ND	69,400[b]	[Bickett, 1993 #1296]
Dnp-Pro-Cha-Gly~Cys(Me)-His-Ala-Lys(Nma)-NH_2	ND	ND	86,600[b]	[Bickett, 1993 #1296]
Dnp-Pro-Cha-Gly~Cys(Me)-His-Ala-Lys(Nma)-NH_2	ND	ND	300,000	[Mohan, 2002 #2459]
Dabcyl-Gaba-Pro-Gln-Gly~Leu-Glu(Edans)-Ala-Lys-NH_2	ND	ND	206,000	[Beekman, 1996 #902]
Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	169,000	[Knight, 1992 #1292]
Mca-Lys-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	623,000[d]	[Neumann, 2004 #2671]
Abz-Gly-Pro-Leu-Gly~Leu-Tyr(NO_2)-Ala-Arg-NH_2	ND	ND	150,000	[Buchardt, 1999 #1847]
Dabcyl-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(Fam)-NH_2	ND	ND	850,000	[Moss, 2007 #3292]
Dabcyl-Leu-Ala-Gln-Ala-Homophe-Arg-Ser-Lys(Fam)-NH_2	ND	ND	220,000	[Moss, 2007 #3292]
α1(V)436–447 fTHP; (Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Pro-Gly~Val-Val-Gly-Glu-Lys(Dnp)-Gly-Glu-Gln-(Gly-Pro-Hyp)$_5$-NH_2	0.044[k]	8.1[k]	5,500[k]	[Lauer-Fields, 2003 #2028]
MMP-11				
Dns-Pro-Leu-Ala~Leu-Trp-Ala-Arg-NH_2	0.012[h]	21[h]	570[h]	[Holtz, 1999 #3301]
Dns-Pro-Leu-Ala~Cys(Mob)-Trp-Ala-Arg-NH_2	0.078[h]	5.2[h]	15,000[h]	[Holtz, 1999 #3301]
MMP-12				
Dnp-Arg-Pro-Leu-Ala-Leu~Trp-Arg-Ser-NH_2	ND	ND	18,000	[Gronski, 1997 #3300]
Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	61,000[d]	[Park, 2000 #1692]
Mca-Pro-Lys-Pro-Leu-Ala-Leu-Dpa-Ala-Arg-NH_2	ND	ND	968,000[d]	[Park, 2000 #1692]
Mca-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys(Dnp)-NH_2	ND	ND	243,000[d]	[Park, 2000 #1692]

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Substrate	k_{cat}[a] (sec^{-1})	K_M[a] (μM)	k_{cat}/K_M[a] (sec^{-1}M^{-1})	Reference
Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2	ND	ND	83,300[d]	[Park, 2000 #1692]
Mca-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Arg-NH_2	ND	ND	13,000[d]	[Park, 2000 #1692]
α1(V)436–447 fTHP; (Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Pro-Gly~Val-Val-Gly-Glu-Lys(Dnp)-Gly-Glu-Gln-(Gly-Pro-Hyp)$_5$-NH_2	2.35[k]	24.8[k]	97,600[k]	[Bhaskaran, 2008 #3295]
MMP-13				
Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	757,000[d]	[Knäuper, 1996 #1324]
Mca-Lys-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	5.9[d]	5.2[d]	1,135,000[d]	[Neumann, 2004 #2671]
Mca-Pro-Cha-Gly~Nva-His-Ala-Dpa-NH_2	ND	ND	1,090,000[d]	[Knäuper, 1996 #1324]
Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	2,520[d]	[Park, 2000 #1692]
Mca-Pro-Lys-Pro-Leu-Ala-Leu-Dpa-Ala-Arg-NH_2	ND	ND	2,680[d]	[Park, 2000 #1692]
Mca-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys(Dnp)-NH_2	ND	ND	408[d]	[Park, 2000 #1692]
Dabcyl-Gly-Pro-Leu-Gly-Met-Arg-Gly-Cys(fluorescein)-NH_2	ND	ND	1,600,000[d]	[Rasmussen, 2004 #2700]
Dabcyl-Gly-Pro-Leu-Gly-Phe-Arg-Val-Cys(fluorescein)-NH_2	ND	ND	2,700,000[d]	[Rasmussen, 2004 #2700]
Dabcyl-Gly-Pro-Ser-Gly-Ile-His-Val-Cys(fluorescein)-NH_2	ND	ND	2,500,000[d]	[Rasmussen, 2004 #2700]
Dabcyl-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(Fam)-NH_2	ND	ND	2,100,000	[Moss, 2007 #3292]
Dabcyl-Leu-Ala-Gln-Ala-Homophe-Arg-Ser-Lys(Fam)-NH_2	ND	ND	460,000	[Moss, 2007 #3292]
fTHP-4; (Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly~Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_5$-NH_2	0.015[k]	8.6[k]	1,600[k]	[Minond, 2006 #2761]
C_{10}-fTHP-4	0.013[k]	3.4[k]	3,800[k]	[Minond, 2006 #2761]
C_{16}-fTHP-4	0.0020[k]	1.4[k]	1,300[k]	[Minond, 2006 #2761]
fTHP-9; (Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly~**Cys(Mob)**-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_5$-NH_2[l]	0.16[k]	6.25[k]	25,000[k]	[Minond, 2006 #2761]
C_{10}-fTHP-9	0.060[k]	3.45[k]	18,000[k]	[Minond, 2006 #2761]
C_{16}-fTHP-9	0.028[k]	2.50[k]	11,000[k]	[Minond, 2006 #2761]
fTHP-10; (Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-**Orn**-Gly~Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_5$-NH_2[l]	0.58[k]	30.1[k]	19,000[k]	[Minond, 2006 #2761]
C_{10}-fTHP-10	0.31[k]	17.4[k]	18,000[k]	[Minond, 2006 #2761]
fTHP-11; (Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-**Orn**-Gly~**Cys(Mob)**-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_5$-NH_2[l]	0.18[k]	13.7[k]	13,000[k]	[Minond, 2006 #2761]
C_{10}-fTHP-11	0.013[k]	7.10[k]	1,900[k]	[Minond, 2006 #2761]
C_{16}-fTHP-11	0.007[k]	7.68[k]	940[k]	[Minond, 2006 #2761]

Substrate	k_{cat}[a] (sec^{-1})	K_M[a] (μM)	k_{cat}/K_M[a] ($sec^{-1}M^{-1}$)	Reference
C_6-fTHP-12; C_6-(Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-**Leu**-Gly~**Met**-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_5$-NH_2[l]	0.060[k]	6.35[k]	9,710[k]	[Minond, 2007 #2768]
fTHP-13; C_n-(Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-**Val**-**Asn**~**Phe**-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_5$-NH_2[l]	0.127[k]	20.5[k]	6,116[k]	[Minond, 2007 #2768]
C_6-fTHP-13	0.060[k]	9.95[k]	5,800[k]	[Minond, 2007 #2768]
MMP-13(Δ249–451)				
Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	639,000[d]	[Knäuper, 1997 #1060]
Mca-Pro-Cha-Gly~Nva-His-Ala-Dpa-NH_2	ND	ND	1,000,000[d]	[Knäuper, 1997 #1060]
MMP-14 catalytic domain				
Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	393,000[d]	[Neumann, 2004 #2671]
Mca-Lys-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	9.9	7.9	1,253,000[d]	[Neumann, 2004 #2671]
Dns-Pro-Leu-Ala~Leu-Trp-Ala-Arg-NH_2	0.67[h]	27[h]	25,000[h]	[Holtz, 1999 #3301]
Dns-Pro-Leu-Ala~Cys(Mob)-Trp-Ala-Arg-NH_2	5.6[h]	8[h]	700,000[h]	[Holtz, 1999 #3301]
Dabcyl-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(Fam)-NH_2	ND	ND	1,900	[Moss, 2007 #3292]
Dabcyl-Leu-Ala-Gln-Ala-Homophe-Arg-Ser-Lys(Fam)-NH_2	ND	ND	73,000	[Moss, 2007 #3292]
MMP-14(Δ279–523)				
fTHP-4; (Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly~Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_5$-NH_2	1.37[k]	23.1[k]	59,000[k]	[Minond, 2006 #2761]
C_{10}-fTHP-4	0.0033[k]	0.80[k]	4,100[k]	[Minond, 2006 #2761]
C_{16}-fTHP-4	0.0021[k]	1.80[k]	1,200[k]	[Minond, 2006 #2761]
fTHP-9; (Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly~**Cys(Mob)**-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_5$-NH_2[l]	1.34[k]	7.95[k]	168,000[k]	[Minond, 2006 #2761]
C_{10}-fTHP-9	0.080[k]	2.80[k]	29,000[k]	[Minond, 2006 #2761]
C_{16}-fTHP-9	0.200[k]	6.60[k]	30,000[k]	[Minond, 2006 #2761]
fTHP-10; (Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-**Orn**-Gly~Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_5$-NH_2[l]	0.25[k]	10.2[k]	25,000[k]	[Minond, 2006 #2761]
C_{10}-fTHP-10	0.091[k]	4.40[k]	20,000[k]	[Minond, 2006 #2761]
fTHP-11; (Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-**Orn**-Gly~**Cys(Mob)**-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_5$-NH_2[l]	1.30[k]	16.6[k]	78,000[k]	[Minond, 2006 #2761]
C_{10}-fTHP-11	0.036[k]	3.50[k]	11,000[k]	[Minond, 2006 #2761]
MMP-14(Δ319–523)				

Substrate	k_{cat}[a] (sec^{-1})	K_M[a] (μM)	k_{cat}/K_M[a] ($sec^{-1}M^{-1}$)	Reference
Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	73,700[d]	[Hurst, 2004 #2356]
fTHP-4; $(Gly\text{-}Pro\text{-}Hyp)_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly~Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-$(Gly\text{-}Pro\text{-}Hyp)_5$-$NH_2$	ND	ND	51,200[d]	[Hurst, 2004 #2356]
MMP-14				
Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	14,600[d]	[Hurst, 2004 #2356]
fTHP-4; C_n-$(Gly\text{-}Pro\text{-}Hyp)_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly~Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-$(Gly\text{-}Pro\text{-}Hyp)_5$-$NH_2$	ND	ND	21,800[d]	[Hurst, 2004 #2356]
MMP-15(Δ268–628)				
fTHP-11; $(Gly\text{-}Pro\text{-}Hyp)_5$-Gly-Pro-Lys(Mca)-Gly-Pro-**Orn**-Gly~**Cys(Mob)**-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-$(Gly\text{-}Pro\text{-}Hyp)_5$-$NH_2$[l]	0.41[k]	14.3[k]	29,000[k]	[Minond, 2006 #2761]
MMP-17 catalytic domain				
Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	0.05[d]	8[d]	5,800[d]	[Wang, 1999 #3299]
Mca-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Arg-NH_2	0.42[d]	26[d]	16,000[d]	[Wang, 1999 #3299]
MMP-25 catalytic domain				
Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	38,000[d]	[English, 2001 #2760]
Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH_2	ND	ND	83,000[d]	[English, 2001 #2760]
MMP-26				
Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	1,850[d]	[Park, 2000 #1692]
Mca-Pro-Lys-Pro-Leu-Ala-Leu-Dpa-Ala-Arg-NH_2	ND	ND	343[d]	[Park, 2000 #1692]
Dnp-Pro-Leu-Gly~Met-Trp-Ser-Arg	ND	ND	9,400[d]	[Park, 2002 #3298]
Mca-Pro-Cha-Gly~Nva-His-Ala-Dpa-NH_2	ND	ND	17,000[d]	[Park, 2002 #3298]
Mca-Pro-Leu-Ala~Nva-Dpa-Ala-Arg-NH_2	ND	ND	30,000[d]	[Park, 2002 #3298]
Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	22,000[d]	[Park, 2002 #3298]
ADAM8				
Dabcyl-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(Fam)-NH_2	ND	ND	2,600[j]	[Moss, 2007 #3292]
Dabcyl-Leu-Ala-Gln-Ala-Homophe-Arg-Ser-Lys(Fam)-NH_2	ND	ND	100,000[j]	[Moss, 2007 #3292]
Dabcyl-His-Gly-Asp-Gln-Met-Ala-Gln-Lys-Ser-Lys(Fam)-NH_2	ND	ND	53,000[j]	[Moss, 2007 #3292]
ADAM10				
Dabcyl-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(Fam)-NH_2	ND	ND	56[j]	[Moss, 2007 #3292]

Substrate	k_{cat}[a] (sec^{-1})	K_M[a] (μM)	k_{cat}/K_M[a] ($sec^{-1}M^{-1}$)	Reference
Dabcyl-Leu-Ala-Gln-Ala-Homophe-Arg-Ser-Lys(Fam)-NH_2	ND	ND	6,200[j]	[Moss, 2007 #3292]
Dabcyl-His-Gly-Asp-Gln-Met-Ala-Gln-Lys-Ser-Lys(Fam)-NH_2	ND	ND	270[j]	[Moss, 2007 #3292]
ADAM12				
Dabcyl-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(Fam)-NH_2	ND	ND	3,000[j]	[Moss, 2007 #3292]
Dabcyl-Leu-Ala-Gln-Ala-Homophe-Arg-Ser-Lys(Fam)-NH_2	ND	ND	280,000[j]	[Moss, 2007 #3292]
Dabcyl-His-Gly-Asp-Gln-Met-Ala-Gln-Lys-Ser-Lys(Fam)-NH_2	ND	ND	40[j]	[Moss, 2007 #3292]
ADAM17 (TACE)				
Mca-Lys-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2	ND	ND	778,400[d]	[Neumann, 2004 #2671]
Mca-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Ala-Arg-NH_2	ND	ND	363,800[d]	[Neumann, 2004 #2671]
Dabcyl-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Ala-Arg-NH_2	ND	ND	466,300[d]	[Neumann, 2004 #2671]
Dabcyl-Leu-Ala-Gln-Ala-Homophe-Arg-Ser-Lys(Fam)-NH_2	ND	ND	430,000[j]	[Moss, 2007 #3292]
Dabcyl-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(Fam)-NH_2	ND	ND	6,800[j]	[Moss, 2007 #3292]
ADAMTS-4-1				
Fam-Ala-Glu~Leu-Gln-Gly-Arg-Pro-Ile-Ser-Ile-Ala-Lys-Tamra	0.86[g]	10.7[g]	80,200[g]	[Wayne, 2007 #3068]
Lys(6-Fam)-Asp-Val-Gln-Glu-Phe-Arg-Gly-Val-Thr-Ala-Val-Ile-Arg-Cys(Qsy-9)-Lys-Gly-Lys	0.22	1.0	215,000	[Hills, 2007 #3042]
ADAMTS-4-2				
fTHPa	0.0120[d]	47.1[d]	415[d]	[Lauer-Fields, 2007 #2649]
fSSPa	0.0720[d]	279.0[d]	258[d]	[Lauer-Fields, 2007 #2649]
Fam-Ala-Glu~Leu-Gln-Gly-Arg-Pro-Ile-Ser-Ile-Ala-Lys-Tamra	2.79[g]	16.2[g]	172,000[g]	[Wayne, 2007 #3068]

[a] Assays were performed at 37 °C, pH 7.5 except where noted. ND = not determined.

[b] Assay performed at 23 °C.

[c] Porcine, not human, MMP.

[d] Assay performed at 25 °C.

[e] Assay performed at pH 6.0.

[f] Assay performed at pH 6.5.

[g] Assay performed at 20 °C, pH 8.0.

[h] Assay performed at 25 °C, pH 6.8.

[i] Assay performed at 22 °C.

[j] Assay performed at 25 °C, pH 8.0.

[k] Assay performed at 30 °C.

[l] Substitutions relative to fTHP-4 are indicated in bold.